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Bandell, Michael; Ansanay, Virginie; Rachidi, Najma; Dequin, Sylvie; Lolkema, Juke S.

Published in:
The Journal of Biological Chemistry

DOI:
[10.1074/jbc.272.29.18140](https://doi.org/10.1074/jbc.272.29.18140)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1997

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Bandell, M., Ansanay, V., Rachidi, N., Dequin, S., & Lolkema, J. S. (1997). Membrane potential-generating malate (MleP) and citrate (CitP) transporters of lactic acid bacteria are homologous proteins - Substrate specificity of the 2-hydroxycarboxylate transporter family. *The Journal of Biological Chemistry*, 272(29), 18140-18146. <https://doi.org/10.1074/jbc.272.29.18140>

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Membrane Potential-generating Malate (MleP) and Citrate (CitP) Transporters of Lactic Acid Bacteria Are Homologous Proteins

SUBSTRATE SPECIFICITY OF THE 2-HYDROXYCARBOXYLATE TRANSPORTER FAMILY*

(Received for publication, March 25, 1997, and in revised form, May 22, 1997)

Michael Bandell‡, Virginie Ansanay§, Najma Rachidi§, Sylvie Dequin§, and Juke S. Lolkema‡¶

From the ‡Department of Microbiology, Groningen Biotechnology and Biomolecular Sciences Institute, University of Groningen, 9751NN Haren, The Netherlands and the §Laboratoire de Microbiologie et Technologie des Fermentations, Institut des Produits de la Vigne, INRA, 2 Place Viala, F-34060 Montpellier Cedex 1, France

Membrane potential generation via malate/lactate exchange catalyzed by the malate carrier (MleP) of *Lactococcus lactis*, together with the generation of a pH gradient via decarboxylation of malate to lactate in the cytoplasm, is a typical example of a secondary proton motive force-generating system. The *mleP* gene was cloned, sequenced, and expressed in a malolactic fermentation-deficient *L. lactis* strain. Functional analysis revealed the same properties as observed in membrane vesicles of a malolactic fermentation-positive strain. MleP belongs to a family of secondary transporters in which the citrate carriers from *Leuconostoc mesenteroides* (CitP) and *Klebsiella pneumoniae* (CitS) are found also. CitP, but not CitS, is also involved in membrane potential generation via electrogenic citrate/lactate exchange. MleP, CitP, and CitS were analyzed for their substrate specificity. The 2-hydroxycarboxylate motif R₁R₂COHCOOH, common to the physiological substrates, was found to be essential for transport although some 2-oxocarboxylates could be transported to a lesser extent. Clear differences in substrate specificity among the transporters were observed because of different tolerances toward the R substituents at the C2 atom. Both MleP and CitP transport a broad range of 2-hydroxycarboxylates with R substituents ranging in size from two hydrogen atoms (glycolate) to acetyl and methyl groups (citromalate) for MleP and two acetyl groups (citrate) for CitP. CitS was much less tolerant and transported only citrate and at a low rate citromalate. The substrate specificities are discussed in the context of the physiological function of the transporters.

The electrochemical gradient of protons across the cytoplasmic membrane is a major store of free energy in the bacterial cell. Usually, the proton motive force (pmf)¹ is generated by translocation of protons against the gradient across the cell membrane which results in the two components of the pmf, a membrane potential and a pH gradient. Proton pumping is catalyzed by primary transport systems at the expense of some

source of chemical energy or light. In certain anaerobes a different mechanism of pmf generation has evolved which involves the action of secondary transporters rather than primary ion pumps and, therefore, is termed secondary pmf generation (for reviews, see Refs. 1 and 2). An example of such a system is the malolactic fermentation pathway found in several lactic acid bacteria (3–5). In *Lactococcus lactis* the uptake of divalent malate is coupled to the exit of its decarboxylation product, monovalent lactate (precursor/product exchange), which leads to the formation of a membrane potential of physiological polarity. Furthermore, the intracellular decarboxylation of malate catalyzed by malolactic enzyme consumes a cytosolic proton which results in a pH gradient over the cell membrane. The combined activities of electrogenic exchange and proton-consuming decarboxylation result in a pmf that is sufficiently high to drive ATP synthesis via the F₀F₁-ATPase (3). Similar pathways have been described for a number of other substrates (6–8). Recently, a more complex system termed citrolactic fermentation was found in *Leuconostoc mesenteroides*, another lactic acid bacterium. Similar to the malolactic fermentation pathway a secondary transporter catalyzes the uptake of divalent citrate in exchange for monovalent lactate, but the pathway in the cytosol converting citrate into lactate requires three different enzymes and is coupled to glucose metabolism (9, 10).

The membrane potential-generating secondary transporters involved in malolactic fermentation and citrolactic fermentation, MleP and CitP, respectively, differ from “usual” secondary transporters in two aspects: (i) they translocate net negative charge across the membrane, and (ii) they catalyze efficient heterologous exchange of two structurally related substrates (the precursor and the product). Functionally, MleP and CitP are quite similar because lactate is a substrate of both, and, moreover, it was shown that malate is a substrate of CitP as well (9). The structural gene coding for CitP was cloned from different organisms (11, 12) and shown to be homologous to the Na⁺-dependent citrate carrier CitS of *Klebsiella pneumoniae* (13). CitS is a usual secondary transporter driven by the pmf and sodium ion motive force (14–16). Recently, CitS was shown to represent a new structural class of secondary transporters with a nine-helix bundle motif (17).

Here, we report the cloning and sequencing of the *mleP* gene coding for the malate transporter of *L. lactis* which is involved in malolactic fermentation. In line with the functional similarities of MleP and CitP the gene was found to be homologous to the *citP* and *citS* genes. The three proteins are part of a family of secondary transporters in which both metabolic energy-dissipating (CitS) and -generating (CitP and MleP) members are found. Essential for MleP and CitP is the ability to transport two differently charged but structurally related molecules,

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¶ To whom correspondence should be addressed: Dept. of Microbiology, Biological Centre, Kerklaan 30, 9751NN Haren, The Netherlands. Tel.: 3150-363-2155; Fax: 3150-363-2154; E-mail: j.s.lolkema@biol.rug.nl.

¹ The abbreviations used are: pmf, proton motive force; ORF, open reading frame; kb, kilobase(s); PCR, polymerase chain reaction; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; COV, cytochrome c oxidase-containing vesicle.

which suggests a wide substrate specificity. It is shown that a broad range of 2-hydroxycarboxylates can be transported by members of the family. In line with its physiological function, the substrate specificity of CitS is much more restricted than observed for the two precursor/product exchangers.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

L. lactis strains IL1403 and IL1441 are wild type malate-fermenting strains; strains MG1363 and LL108 do not ferment malate. Strain IL1441 is a streptomycin-resistant derivative of IL1403 (18). Strain LL108, kindly provided by K. Leenhouts, is a chloramphenicol-resistant derivative of MG1363 with multiple copies of the *repA* gene inserted in its chromosome which results in an increase in plasmid copy number (19). *L. lactis* NCDO176 is a wild type citrate-fermenting strain obtained from the Dutch Institute of Dairy Research (Ede, The Netherlands). The *L. lactis* strains were grown in closed serum bottles without shaking in M17 broth (Difco) supplemented with 0.5% (w/v) glucose and at 30 °C. Concentrations of 5 µg/ml erythromycin and 5 µg/ml chloramphenicol were used when indicated. *L. mesenteroides* ssp. *mesenteroides* 19D was grown at 30 °C in MRS medium without acetate and Tween and with 0.5% ammonium citrate (20). *Escherichia coli* strains DH5α, BL21(DE3), and MC1061 were grown aerobically at 37 °C in Luria broth supplemented with 100 µg/ml carbenicillin or 10 µg/ml chloramphenicol when indicated.

Recombinant DNA Techniques

Standard DNA manipulation techniques were carried out essentially as described by Sambrook *et al.* (21). *L. lactis* genomic DNA was isolated as described by Simon *et al.* (22). *L. lactis* and *L. mesenteroides* plasmid DNA was isolated as described by Leenhouts *et al.* (23). *L. lactis* was transformed by electroporation as described by Holo and Nes (24). For sequencing, plasmids were transformed to and propagated in *E. coli* DH5α. Nucleotide sequences were determined on a Vistra 725 or Applied Biosystems 373A automated sequencer.

Cloning and Sequencing of the *mleP* Gene

Plasmid p153A has been described before (25) and was shown to contain *mleS* encoding malolactate enzyme and the 5' part of a second open reading frame ORF2, presumably *mleP* encoding the malate transporter. A 3-kb fragment obtained by *Bam*HI digestion of chromosomal DNA isolated from *L. lactis* IL1441 overlapped with the insert in p153A. The fragment was cloned into the unique *Bam*HI restriction site of plasmid pUC18 (26) yielding p191A. Unidirectional sets of nested deletions of p191A were constructed using exonuclease III (Pharmacia Biotech Inc.). Truncated fragments were sequenced using vector-specific primers after which internal primers were designed to sequence the remaining regions of each strand. The insert contained the first 1,068 base pairs of ORF2. To clone the missing 3' end of ORF2, an *Eco*RI/*Bam*HI fragment of p191A containing the final 900 base pairs of the ORF2 fragment was used to probe a *Hind*III digest of chromosomal DNA. The sequence of a 2.5-kb fragment that hybridized with the probe was determined using inverted polymerase chain reaction (PCR). Genomic DNA of *L. lactis* IL1441 digested with *Hind*III was ligated with T4 DNA ligase under conditions that favored the formation of monomeric circles. The circular fragments were used as a template for a PCR using oligonucleotides based on the sequence of the *Eco*RI/*Bam*HI fragment of p191A. The primer sequences were 5'-TGGCAGG-TATTGGTCTTG-3' and 5'-CCATACCACCTGACATCAT-3'. A 2-kb fragment presenting a head to tail structure was amplified and cloned into pGEM-T (Invitrogen) to give vector pPNJ. The missing 0.4-kb fragment of ORF2 on pPNJ was sequenced on both strands. ORF2 was amplified from genomic DNA using a forward primer based on the sequence of p153A (5'-ATCTCGAGATGAAAAAACTTAAAGAA-3') and a reverse primer based on the sequence of pPNJ (5'-GATAGCGGATAT-AGTCCC-3') and ligated into pGEM-T, yielding pPME. The sequence of ORF2 reconstructed from all of the fragments was confirmed by sequencing one strand of the pPME insert.

Construction of Expression Vectors

An expression vector was constructed containing a promoter region that is located in front of the *citP* gene on the 7.9-kb plasmid of *L. lactis* NCDO176 (27). The 1,572-base pair region was amplified from the plasmid by PCR. The forward primer, 5'-GCTTAGAATTCTTGCTAT-CAGTATGTC-3', introduced an *Eco*RI restriction site in the fragment. The reverse primer, 5'-GGTGGATCCACATGTTTCTATCTCCATT-

TATATC-3', overlapped with the *citP* start codon and introduced a unique *Afl*III site (ACATGT) around the start codon and a *Bam*HI site at the end. Bases introducing mutations are underlined, and the start codon is indicated in bold. The PCR product was digested with *Eco*RI and *Bam*HI and ligated into plasmid pGK13 (28) digested with the same enzymes. The resulting plasmid pMB contains the *L. lactis citP* promoter region followed by a ribosomal binding site and an *Afl*III restriction site around the start codon. Downstream of the *Afl*III restriction site a number of unique restriction sites are present for cloning purposes.

The gene coding for the malate transporter *mleP* was amplified by PCR from chromosomal DNA isolated from *L. lactis* IL1403. The forward primer, 5'-TCTTAATCCATGGGAAAAAACTTAAAGAAAC-GAAAATATCGG-3', generated an *Nco*I site around the start codon, and the reverse primer, 5'-TGCTCTAGATTACGCGTATACAAAGAATCG-GATAAGAATTCCACC-3', generated an *Xba*I site downstream of the stop codon. Start and stop codons are indicated in bold. Similarly, the gene coding for the citrate transporter *citP* was amplified from an endogenous plasmid preparation of *L. mesenteroides* while creating unique *Nco*I and *Xba*I restriction sites around the start codon and downstream of the stop codon, respectively. The forward primer was 5'-GATAGAACCATGGTGAATCACCCGCATTC-3' and the reverse primer 5'-CTTTAAATATCTAGATTACTTTCATG-3'. The amplified fragments were digested with *Nco*I and *Xba*I and ligated into the *Afl*III and *Nhe*I sites of pMB which have compatible overhangs. The resulting vectors pMB*mleP* and pMB*citP* code for *mleP* and *citP* under control of the *citP* promoter. In case of MleP, the cloning procedure resulted in a Gly insertion after position 1, and the COOH-terminal Tyr residue was replaced by Val-Tyr-Ala. In CitP Met2 was replaced by Val. The sequence of the inserts was confirmed by automated sequencing.

Preparation of Membrane Vesicles and Hybrid Membranes

Cells of *L. lactis* MG1363 or LL108 expressing either MleP or CitP were harvested at the end of the exponential growth phase at an A_{660} of 0.8, washed with 50 mM potassium phosphate, pH 7.0, resuspended in the same buffer at an A_{660} of 500, and subsequently rapidly frozen in liquid nitrogen until use. Rightside-out membrane vesicles were prepared by the osmotic shock lysis procedure essentially as described by Otto *et al.* (29). The membranes were fused to liposomes or to proteoliposomes containing beef heart cytochrome *c* oxidase (COVs) essentially as described by Driessen *et al.* (30). Liposomes consisted of a mixture of purified *E. coli* lipids and egg yolk phosphatidylcholine at a ratio of 3:1. *E. coli* lipids were purified by successive washing of an *E. coli* extract (Avanti Polar Lipids) with acetone and diethyl ether, after which the concentration was determined as described by Driessen *et al.* (31). Cytochrome *c* oxidase isolated from beef heart mitochondria was reconstituted into liposomes by detergent dialysis. Liposomes or COVs were fused with the membrane vesicles of *L. lactis* at a ratio of 10 mg of lipid and 1 mg of protein by a single freeze/thaw step (30). The buffer contained 50 mM potassium phosphate, pH 6. In case of fusion with liposomes 5 mM L-malate or citrate was included in the buffer to load the vesicles. The resulting hybrid membranes were made unilamellar by subsequent extrusion through 400- and 200-nm pore size polycarbonate filters (32). Hybrid membranes were concentrated by ultracentrifugation at $250,000 \times g$ for 20 min at 10 °C.

Rightside-out membrane vesicles of *E. coli* strain BL21(DE3) expressing CitS from pSKΔ*LcitS* (17) were prepared by the osmotic lysis procedure essentially as described by Kaback (33) with the following modification. Spheroplasts were lysed in 50 mM potassium phosphate, pH 7, containing 5 mM potassium citrate to load the vesicles with citrate. All subsequent steps were done in the presence of 5 mM potassium citrate. Before use the membrane vesicles were washed once in 15 volumes of potassium phosphate, pH 6, containing 5 mM citrate and appropriate concentrations NaCl and KCl and concentrated by centrifugation for 20 min in a Eppendorf tabletop centrifuge operated at full speed. Protein concentrations were determined as described by Lowry *et al.* (34).

Transport Assays

Pmf-driven Uptake in Hybrid Membranes—The experiments were performed in 50 mM potassium phosphate, pH 6.0, under a flow of water saturated air and continuous stirring at 30 °C. Membrane vesicles fused with COVs were incubated for 1 min in the presence of 200 µM TMPD, 20 µM cytochrome *c* (horse heart, Sigma), and 10 mM potassium ascorbate. The assay volume was 100 µl and the membrane protein concentration 0.6–0.8 mg/ml. Valinomycin and nigericin were used at concentrations of 1 µM and 0.5 µM, respectively. L-[1,4(2,3)¹⁴C]malate or

MleP	MK-----LKETKISGISLPLVAFVAVITVVTLGK	32
CitP	MMNHPHSSHIGTTNVKEEIGKLDRIISGIGLVRYAFMAVLLTIAISTKT	50
CitS	MTNMSQPP----ATEKKGVSDDLGFKIFGMPLPLVAFALITLLLSHFYNA	46
	* * * * *	
MleP	LPLDMVGLTLLLVTLGHLLYFICEKFPIMNSYLGGGSVFTLIGATLLSFF	82
CitP	LPNTMIGAFALVLMGHVFYYLGAHLPIFRSYLGGGSVFTILLTALVAT	100
CitS	LPTDIVGGFAIMFIICAIIGEIGKRLPIFNKVIIGCAPVMIFLVAYFYVA	96
	** * * *	
MleP	HTVPSNVIGAVSNFMGGKFGFLDFYIAALICGSILGMNRLNLLVAKSKKFI	132
CitP	NVMPKYVVTASGEINGM-DFLGLYIVSLTIASSLFKMDRKMLLKAARFL	149
CitS	GIFTQKEIDAIISNMD-KSNFLNLFIAVLLTGAILSVNRLLLRSLGTY	145
	* ** * *	
MleP	PIALITMVIGFFSVGLVGMILIGNGFADSVTVSMPMMSGMGAGITPLSQ	182
CitP	PVAFISMALTAVVIGIVGVIIIGVGFNYAILMIAMPIMAGGVGAGIVPLSG	199
CitS	PTILMGIVGASIFGIAIGLVFGIPVDRIMMLYVLPIMGGGNGAGAVPLSE	195
	* * * * *	
MleP	IYAAGLAHGNQAAIFSOIAPAVTFGNILATIGALSTAKVFNKSK-YNGHG	231
CitP	IYAHAMGVGS-AGILSKLFFFTVILGNLLAILSAGLISRIKFSK-GNGHG	247
CitS	IY-HSVTGRSREEYYSATAILTIANIFAIVFAAVLDIIGKKHTWLSGEG	244
	** * * *	
MleP	TLVAATKEELAKP--KIKLDAQOICTGMLFAFPLLMAGDIL-NKFFPNTI	277
CitP	EILRGEREDAAAAAEEKPDYVQLGVGLIIVMFFMIGTML-NKVFPGI	295
CitS	ELVRKASFKVEDEKGTQITHRETAAGLVLTTCFLLAYVVAKKILPSIG	294
	* * *	
MleP	----HQYAFMTIIVFILKATNTVPKDLEESVVMFNQVIMTNLTHAVLAGI	323
CitP	----NAYAFIILSIVLTKAFGLPKYYEDSVIMFGQVIVKNMTHALLAGV	341
CitS	GVATHYFAWMVLIIVAAINASGLCSPEIKAGAKRLSDFFSKQLLWVLMGV	344
	* * *	
MleP	GLAIDIDINTLASAFTWQFVVLCLTSVVVMGLASWFLARLFGLYPVETAGI	373
CitP	GLSLDMHVLAAALSQFVVLCLTSVIVASISLISATLGKLFGLYPVEAAT	391
CitS	GVCYTDLQEIINAITFANVIAAIIIVIGAVLGAAGWLMGFFPIESAIT	394
	* * * * *	
MleP	AGMINNSMGGTGNIAVLSASDRMEMTAFQAQMANRLCGAIVLIFGGIILRE	423
CitP	AGLANNNSMGGTGNVAVLAASERMNLTAFAQMCNRIGGALILVVGILVTF	441
CitS	AGLCMANRGGSGDLEVLACNRMNLISYAQITSSRLGGGIVLVIASTVFGM	444
	** * * * *	
MleP	FY	425
CitP	MK	443
CitS	MI	446

FIG. 1. Alignment of the primary sequences of MleP, CitP, and CitS. Identical residues are printed in gray boxes. Residues conserved in all three transporters are indicated by an asterisk. Putative transmembrane segments (17) are indicated by bars.

[1,5-¹⁴C]citrate was added at a concentration of 13.1 or 4.4 μ M, respectively. Uptake was stopped at different time intervals by adding 2 ml of ice-cold 0.1 M LiCl to a sample and rapid filtration over 0.45- μ m pore size cellulose nitrate filters (Schleicher & Schuell). Filters were rinsed once with 2 ml of ice-cold 0.1 M LiCl and transferred to scintillation vials, and the internalized radioactivity was determined.

Exchange in Membrane Vesicles—Membrane vesicles of *L. lactis* LL108 fused to liposomes and *E. coli* rightside-out membrane vesicles preloaded with 5 mM L-malate or citrate were concentrated by centrifugation. Strain LL108 was used for this assay because of the higher expression of MleP and CitP in this strain. Concentrated hybrid membranes were incubated in 50 mM potassium phosphate, pH 6, with 50 μ M nigericin with 72.5 μ M [1,5-¹⁴C]citrate or 186.7 μ M L-[1,4(2,3)-¹⁴C]malate for 30 min at room temperature. When indicated, 100 μ M valinomycin or 100 mM KSCN was present in the assay mixture. Similarly, the concentrated *E. coli* membranes were incubated in 50 mM potassium phosphate, pH 6, in the presence of 100 μ M valinomycin and 50 μ M nigericin with 217.5 μ M [1,5-¹⁴C]citrate and the appropriate concentrations of NaCl and KCl for 2 h. Aliquots of 2 μ l were diluted 100-fold into buffer of 20 C containing various substrates at a concentration of 5 mM. The buffer contained 100 mM KCl in the case the membranes where

preloaded with KSCN. Final membrane protein concentrations in the assays were 0.1–0.15 mg/ml and 0.23 mg/ml for *L. lactis* hybrid membranes and *E. coli* membranes, respectively. Samples were stopped and processed as described above. The data were fitted to an exponential decay. Within one set of experiments, the zero time point was determined from the curve representing efflux which is slow enough to allow a linear back extrapolation. The infinite time point was estimated from curves representing rapid exchange, usually homologous exchange.

Chemicals

[1,5-¹⁴C]Citrate (115 mCi/mmol) and L-[1,4(2,3)-¹⁴C]malate (51 mCi/mmol) were obtained from Amersham International (Buckinghamshire, U. K.). Oligonucleotides were obtained from Eurosequence (Groningen, The Netherlands) and from Eurogentec (Seraing, Belgium). All other compounds were obtained from commercial sources.

RESULTS

Cloning and Sequencing of mleP—In a previous paper the cloning and sequencing were reported for the gene *mleS* encoding malolactic enzyme, the decarboxylase in the malolactic

fermentation pathway of *L. lactis* (25). One of the clones contained the 5' end of a second ORF starting 15 base pairs downstream of the stop codon of *mleS*. It was suggested that this ORF would be *mleP* coding for the malate transporter, the second protein of the pathway. The complete second reading frame, 1,278 base pairs in length, was cloned as described under "Experimental Procedures," and the nucleotide sequence was determined. A putative Shine-Dalgarno sequence AAGG is found 16 nucleotides upstream of the start codon (ATG). The stop codon (TAA) is followed closely by an inverted repeat that has features typical of a putative rho-independent transcription termination signal (35). This organization suggests that the two genes are organized in an operon. To verify this, total RNA was isolated from *L. lactis* IL1441 grown in medium on glucose with and without additional malate. Malolactic enzyme coded by *mleS* is an inducible enzyme (18). The results demonstrated that in malate-grown cells a single transcript of approximately 3 kb hybridized with a probe specific for *mleP* and with plasmid p191A containing *mleS* and a 5' fragment of *mleP*. No band was detected when malate was omitted from the growth medium (not shown). The *mleP* sequence is available under accession number X75982.

Analysis of the deduced MleP amino acid sequence reveals a hydrophobic protein of 425 amino acid residues with a predicted mass of 46.7 kDa. Screening of the available data bases showed that the protein revealed homology to the citrate transporters of lactic acid bacteria (CitPs) and to the Na⁺-dependent citrate carriers of *K. pneumoniae* (CitS), *Salmonella pullorum* (CitC), and *Salmonella dublin* (CitC). Like the lactococcal CitPs, the Na⁺-dependent transporters form a group of proteins with almost identical primary sequences (>95%). The alignment of MleP with representative sequences from these

two groups is shown in Fig. 1. MleP is most similar to CitP with 48% identical residues and shares 30% sequence identity with CitS. Overall, the alignment shows 86 (19%) conserved residues with an additional 85 similar residues. A glycine-rich region around residue 175 in MleP and approximately the COOH-terminal 60 residues are the most conserved regions in the family. Fig. 2 shows the hydrophathy profiles of the individual members (*thin lines*) and the average profile of the family (*bold*). The profiles are remarkably similar, indicative of the same global structure.

Functional Expression of MleP—To determine whether the cloned gene identified as *mleP* is the malate transport protein involved in malolactic fermentation, the gene was expressed in *L. lactis* MG1363 and LL108, strains not able to ferment malate. Cytoplasmic membranes with a rightside-out orientation prepared from *L. lactis* MG1363 harboring pMB*mleP* (see "Experimental Procedures") were fused to proteoliposomes reconstituted with purified beef heart cytochrome *c* oxidase (COVs) as a pmf-generating system. In these hybrid membranes a pmf (inside negative and alkaline relative to the outside) is generated in the presence of the electron donor system potassium ascorbate, TMPD, and cytochrome *c*. In the presence of a pmf the hybrid membranes took up a low but significant amount of [¹⁴C]malate (Fig. 3A, ○). Control experiments with hybrid membranes prepared from membrane vesicles of strain MG1363 without pMB*mleP* showed no uptake under identical conditions (not shown). Therefore, the product of the *mleP* gene is a malate transporter. The pmf generated by cytochrome *c* oxidation is composed of a membrane potential ($\Delta\psi$) and a pH gradient (ΔpH). The role of each component of the pmf in driving [¹⁴C]malate uptake was investigated by manipulating ΔpH and $\Delta\psi$ with the ionophores nigericin, a K⁺/H⁺ antiporter, and valinomycin, a K⁺ pore. In the presence of nigericin, when the pmf consists solely of a membrane potential, no uptake was observed, indicating that the membrane potential is not a driving force for malate transport (■). On the other hand, in the presence of valinomycin, when the pmf is composed solely of a pH gradient, a strong stimulation of malate uptake was observed, indicating that the membrane potential counteracts malate transport (▲) and that net negative charge is translocated across the membrane during turnover.

A second important feature of the malate carrier involved in malolactic fermentation is the physiological mode of transport, i.e. heterologous malate/lactate exchange (3). Rightside-out membrane vesicles of *L. lactis* LL108 harboring pMB*mleP* were fused with liposomes and preloaded with 5 mM L-[¹⁴C]malate. 100-fold dilution of the membranes in buffer did not result in significant release of label within the first 40 s, indicating that efflux of malate down a concentration gradient is a slow process

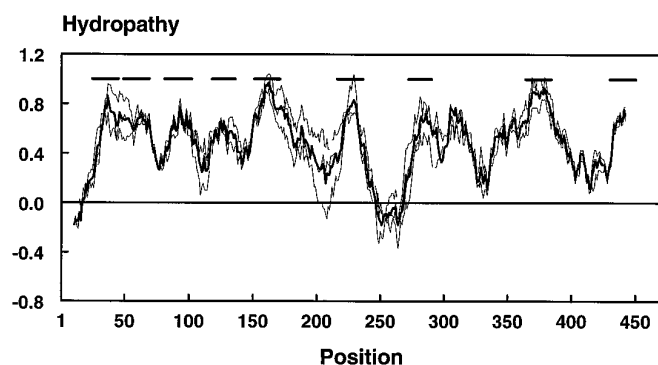


FIG. 2. Hydrophathy profile of MleP, CitP, and CitS. The individual profiles (*thin*) and average profile (*bold*) were calculated with a window of 21 residues and the normalized hydrophobicity scale of Kyte (42). Putative transmembrane segments are indicated by bars (17).

FIG. 3. Malate uptake in the presence of a pmf (panel A) and exchange (panels B and C) catalyzed by MleP. Panel A, malate (13.1 μM) uptake by membrane vesicles of MG1363/pMB*mleP* fused with COVs was assayed in the presence of the electron donor system cytochrome *c*/TMPD/potassium ascorbate. ○, no ionophores; ▲, valinomycin; and ■, nigericin. Panels B and C, membrane vesicles of *L. lactis* LL108 expressing MleP, fused with liposomes were preloaded with 5 mM L-[¹⁴C]malate with (panel C) and without (panel B) 100 mM KSCN and subsequently diluted 100-fold into buffer containing 5 mM lactate (□), malate (▲), or no additions (○). Valinomycin (panel B) and nigericin (panels B and C) were present at 1 and 0.5 μM , respectively.

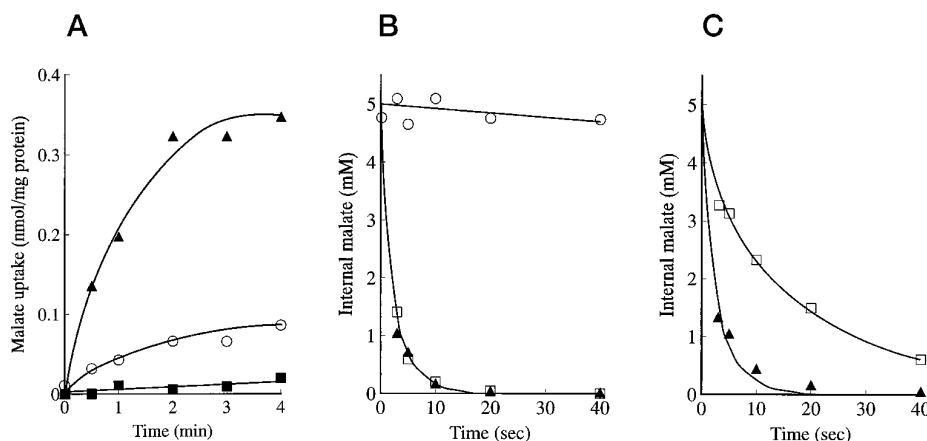


FIG. 4. Effect of the sodium ion concentration on homologous exchange and efflux catalyzed by CitS. RSO membrane vesicles of *E. coli* BL21(DE3) expressing CitS were preloaded with 5 mM [14 C]citrate and with no further additions (panel A), with 1 mM NaCl (panel B), and with 75 mM NaCl (panel C). The Cl^- concentration was kept constant by adding compensating amounts of KCl. The membranes were diluted 100-fold into buffer containing the same NaCl and KCl concentrations, in the presence (\blacktriangle) or the absence (\circ) of 5 mM citrate. Valinomycin and nigericin were present at 1 and 0.5 μM , respectively.

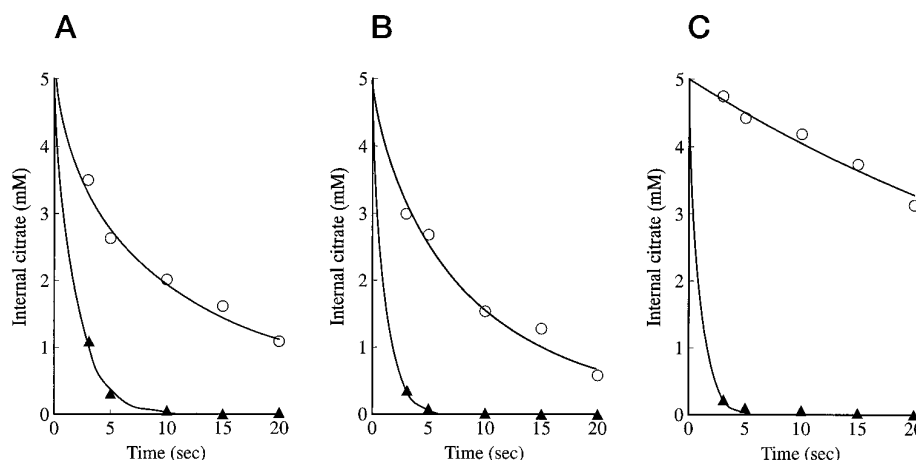


TABLE I
Effect of the R substituents on the ability of MleP, CitP, and CitS to transport 2-hydroxycarboxylates

Substrate ^a	R ₁	R ₂	Relative rate of exchange ^b		
			MleP	CitP	CitS
				%	
Isocitrate	$\text{CHCOO}^- \text{CH}_2\text{COO}^-$	H	0.4 ± 0.2	2.6 ± 0.4	1.5 ± 0.7
Citrate	CH_2COO^-	CH_2COO^-	0.5 ± 0.3	100 ± 8	100 ± 6
Citromalate	CH_2COO^-	CH_3	6.9 ± 1.0	32 ± 1	4.6 ± 0.5
Tartrate	CHOHCOO^-	H	18 ± 1	7.1 ± 0.2	0.9 ± 0.5
Malate	CH_2COO^-	H	100 ± 8	142 ± 6	1.6 ± 0.8
2-Hydroxybutyrate	CH_2CH_3	H	15 ± 3	54 ± 1	1.3 ± 0.4
2-Hydroxyisobutyrate	CH_3	CH_3	16 ± 3	100 ± 1	1.9 ± 0.7
Lactate	CH_3	H	91 ± 14	36 ± 1	1.7 ± 0.5
Glycolate	H	H	26 ± 8	3.3 ± 0.5	1.7 ± 0.8
None			0.5 ± 0.3	1.0 ± 0.5	1.0 ± 0.6

^a Substrates were added at a concentration of 5 mM. Equal mixtures of the L- and D-isomers were used.

^b Rates are relative to the rate observed for homologous exchange, which was set at 100%. These rates varied per vesicle preparation between 2.7 and 4.8, 0.7 and 0.8, and 2.3 and 2.6 mM/s for MleP, CitP, and CitS, respectively. Rates are given as the average of two to four independent measurement and the S.D.

(Fig. 3B, \circ). In contrast, dilution of the preloaded membranes in buffer containing an equimolar concentration of unlabeled malate resulted in rapid release of internal labeled malate, indicative of rapid homologous exchange (\blacktriangle). Most importantly, the same rapid release of label was observed upon dilution into buffer containing an equimolar concentration of lactate (\square). To prevent the formation of ΔpH or $\Delta\psi$ which would counteract efflux, the ionophores valinomycin and nigericin were included in these experiments. Preloading of the membranes with the membrane permeable ion SCN^- results in the generation of a diffusion potential, negative outside, upon dilution when valinomycin is omitted from the assay mixture. The diffusion potential significantly inhibited malate/lactate exchange whereas malate/malate exchange was not affected (Fig. 3C) showing that heterologous exchange is electrogenic. The results are consistent with those found for malate transport in membrane vesicles of the wild type malate-fermenting *L. lactis* IL1403 (3) showing that the *mleP* gene product is the malate transporter involved in malolactic fermentation.

Substrate Specificity of MleP, CitP, and CitS—The heterologous exchange assay demonstrated in Fig. 3B provides a sensitive and unambiguous assay for the substrate specificity of a transporter in general and was used to determine the substrate specificity of MleP and the two other representatives of the family, CitP of *L. mesenteroides* and CitS of *K. pneumoniae*. To use the assay it is essential that efflux is much slower than exchange. This condition is a property of precursor/product exchangers (2) and has also been demonstrated for CitP (9). In case of CitS, conditions of rapid exchange/slow efflux were sought by varying the concentration of the symported Na^+ ion

(Fig. 4). With no additional Na^+ added, efflux and exchange in rightside-out membrane vesicles of *E. coli* BL21(DE3) harboring plasmid pSK ΔcitS (17) were observed at comparable rates. Since no effort was made to work “sodium free” the observed activities are most likely caused by sodium ion contaminations in the buffer (15). Addition of 1 mM Na^+ increased both the rate of efflux and exchange. However, at 75 mM added Na^+ the rate of exchange increased further, but the rate of efflux decreased. This behavior is typical for a solute/co-ion symporter (36). The latter condition was used for the substrate specificity assay.

From previous studies it was known that MleP transports both malate and lactate, CitP transports citrate, lactate, and malate (9), whereas for CitS no substrate other than citrate has been reported. These substrates all share the 2-hydroxycarboxylate motive, $\text{R}_1\text{R}_2\text{COHCOO}^-$. The effect of the R_1 and R_2 groups, the hydroxyl group, and the carboxylate group on the ability of the three transporters to translocate the substrates was investigated subsequently.

The R Substituents—Nine different 2-hydroxycarboxylates with R substituents which differ both in size and polarity were included in the assay. The results are summarized in Table I. The main conclusion is that CitS is very specific, transporting only citrate and to a low extent citromalate, whereas MleP and CitP transport a wide variety of these substrates. MleP has a preference for the smaller substrates, whereas CitP seems to prefer the larger molecules. In contrast to CitP, MleP does not transport citrate and only poorly citromalate, whereas CitP even transports isocitrate, which has the largest substituent, at a low but significant rate. At the other end of the spectrum, glycolate is a good substrate of MleP and a poor substrate of

TABLE II
Effect of OH substituents on the ability of MleP, CitP, and CitS to transport a substrate

Details are in the legend to Table I.

Substrate	OH substituent	Relative rate of exchange		
		MleP	CitP	CitS
		%		
Citrate	OH	0.5 ± 0.3	100 ± 8	100 ± 6
Tricarballate	H	1.0 ± 0.2	2.2 ± 0.7	1.0 ± 0.1
Malate	OH	100 ± 8	142 ± 6	1.6 ± 0.8
Succinate	H	0.4 ± 0.1	0.5 ± 0.3	1.0 ± 0.5
Oxaloacetate	O	27 ± 4	14 ± 1	1.5 ± 0.8
Aspartate	NH ₃ ⁺	0.7 ± 0.3	1.3 ± 0.3	0.8 ± 0.5
Lactate	OH	91 ± 14	36 ± 1	1.7 ± 0.5
Propionate	H	0.5 ± 0.1	0.7 ± 0.3	1.3 ± 0.4
Pyruvate	O	1.2 ± 0.3	4.2 ± 0.7	1.9 ± 0.8
Alanine	NH ₃ ⁺	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.4
Glycolate	OH	26 ± 8	3.3 ± 0.5	1.7 ± 0.8
Acetate	H	0.5 ± 0.3	0.3 ± 0.2	1.0 ± 0.5
Glyoxylate	O	13 ± 2	2.2 ± 0.2	0.9 ± 0.5
Glycine	NH ₃ ⁺	0.7 ± 0.3	1.1 ± 0.3	0.9 ± 0.5
2-Hydroxybutyrate	OH on C2	15 ± 3	54 ± 1	1.3 ± 0.4
3-Hydroxybutyrate	OH on C3	1.1 ± 0.5	3.9 ± 0.2	1.5 ± 0.6
None		0.5 ± 0.3	1.0 ± 0.5	1.0 ± 0.6

CitP. No clear discrimination is evident between substituents with a polar and a hydrophobic character.

The Hydroxyl Group—A set of compounds was selected in which the hydroxyl group of one of the transported substrates listed in Table I was replaced by another substituent. In line with the high specificity of CitS reported above, none of these compounds was transported by CitS (Table II). Substrates in which the hydroxyl group is replaced by a hydrogen atom are not transported. Similarly, the hydroxyl cannot be replaced by an amino group. Replacement of the hydroxyl group by a keto group resulted in significant transport in the case of oxaloacetate, especially by MleP. This transporter could also transport glyoxylate, the 2-oxo analog of glycolate, at a significant rate, whereas the rate catalyzed by CitP was very low but significant. The latter was also observed for pyruvate, the 2-oxocarboxylate analog of lactate, for both MleP and CitP. The position of the hydroxyl group relative to the carboxylate group was investigated by comparing 2-hydroxybutyrate and 3-hydroxybutyrate. A low but significant transport activity with the latter substrate was observed with CitP but not with MleP. In conclusion, MleP and CitP have the highest activity with 2-hydroxycarboxylates, but significant activity is observed with some 2-oxocarboxylates and in case of CitP even a 3-hydroxycarboxylate.

The Carboxylate Group—Three different analogs of lactate with different substituents replacing the carboxylate group were tested in the exchange assay (Table III). A common feature of the analogs 1,2-propanediol, methyl lactate, and glyceraldehyde is that the charge of the carboxylate is removed. In addition to the aldehyde group replacing the carboxylate, glyceraldehyde has a hydroxyl group at the C3 position. This might have an additional effect on the suitability as a substrate, but, on the other hand, tartrate, an analog of malate, has the same feature and is transported both by MleP and CitP (Table I). None of the analogs was transported by any of the transporters, emphasizing the relevance of the carboxylate group in the motif.

DISCUSSION

Secondary pmf-generating pathways were discovered only in the last decade. Oxalate decarboxylation in *Oxalobacter formigenes* and malolactic fermentation in *L. lactis* were the first

TABLE III
Effect of COO[−] substituents on the ability of MleP, CitP, and CitS to transport a substrate

Details are in the legend to Table I.

Substrate	COO [−] substituent	Relative rate of exchange		
		MleP	CitP	CitS
		%		
Lactate	COO [−]	91 ± 14	36 ± 1	1.7 ± 0.5
Methyl-L-lactate	COOCH ₃	1.0 ± 0.1	0.8 ± 0.1	1.3 ± 0.4
1,2-Propanediol	CH ₂ OH	0.4 ± 0.3	0.4 ± 0.2	1.3 ± 0.4
Glyceraldehyde	CHO	0.9 ± 0.5	0.7 ± 0.5	1.1 ± 0.5
None		0.5 ± 0.3	1.0 ± 0.5	1.0 ± 0.6

systems described in detail (3, 6). Both pathways consist of only two enzymes, a secondary transporter and a cytoplasmic decarboxylase. The secondary transporters that take up the substrate in exchange for the decarboxylation product (precursor/product exchange) and, thereby, generate the membrane potential play a central role in the pathways. Recently, the structural gene coding for the oxalate/formate exchanger OxlT of *O. formigenes* was cloned and sequenced (37). Here, we report the cloning and sequencing of MleP, the malate/lactate exchanger of *L. lactis*. The cloned gene was expressed in the malolactic fermentation-negative *L. lactis* strains MG1363 and LL108. Functional characterization in membrane vesicles derived from these cells showed that the gene product conferred the same transport characteristics as was observed before in membrane vesicles of the malate-fermenting wild type strain IL1403 (3). The cloned transporter catalyzed efficient heterologous malate/lactate exchange, and unidirectional uptake into the membranes was counteracted by the membrane potential.

The *mleP* gene of *L. lactis* is not homologous to the *oxlT* gene of *O. formigenes*, indicating that genes coding for membrane potential-generating secondary transporters do not form a separate gene family. MleP was found to be homologous to the membrane potential-generating citrate transporter CitP of lactic acid bacteria and the Na⁺-dependent citrate transporters CitS (13) and CitC (38) of *K. pneumoniae* and *Salmonella* species. The homology to CitS and CitC which are metabolic energy-dissipating transporters suggests that MleP and CitP, and membrane potential-generating secondary transporters in general, are conventional secondary transporters. The membrane topology of CitS was recently reported to be quite different from the transmembrane 12-helix motif usually observed for secondary transporters (17). CitS traverses the membrane 9 times (the bars in Fig. 1) with a cytoplasmic amino terminus and a periplasmic carboxyl terminus. The sequence homology and the highly conserved hydrophathy profile of the members in the family strongly suggest that MleP and CitP fold in a similar fashion in the membrane. In this structural model the two most conserved regions in the alignment shown in Fig. 1 are located in the periplasmic loop between helices V and VI and the cytoplasmic loop preceding the COOH-terminal helix IX. The alignment shows 6 conserved positively charged amino acid residues of which only Arg-407 (MleP numbering) is predicted to be located in the membrane, in putative helix IX. Since MleP, CitP, and CitS transport negatively charged substrates this Arg residue could play a role in substrate binding and/or transport.

Previous studies had shown that MleP, CitP, and CitS transport one or more of the structurally related substrates citrate, malate, and lactate (3, 9, 14), and it was noted that these substrates all contain the motif R₁R₂COHCOO[−] (9). In the present study the importance of the hydroxyl and carboxylate groups of the substrates was investigated. None of a limited number of lactate analogs in which the carboxylate group was

methyated or replaced by an hydroxyl or aldehyde group could be translocated by any of the transporters. This suggests that the carboxylate and possibly the negative charge of this group are essential. A larger number of analogs showed that replacement of the hydroxyl group by a hydrogen atom or an amino group completely abolished transport activity (Table II). However, replacement by a keto group resulted to some extent in activity with MleP and CitP, especially with oxaloacetate. Moving the hydroxyl to the C3 position resulted in a low transport activity by CitP. Possibly, hydrogen bonding between a residue on the transporter and the hydroxyl or keto group on the substrates is essential for translocation. The transport activity of CitP with oxaloacetate is remarkable since oxaloacetate is the first metabolic intermediate in the citrate degradation pathway in lactic acid bacteria (39). Since the 2-hydroxycarboxylates are the physiological and preferred substrates of these carriers we have termed the family the 2-hydroxycarboxylate transporter family.

A typical feature of membrane potential-generating secondary transporters like MleP and CitP is the ability to translocate two structurally related substrates, *i.e.* malate/lactate and citrate/lactate, respectively. The transporters specifically recognize the common 2-hydroxycarboxylate motif. At the same time, MleP and CitP need to be quite tolerant toward the R₁ and R₂ groups since the cytoplasmic conversion of the substrate into the product not only results in a smaller molecule but also removes the charge on one of the R groups. Charge removal is crucial and results in the generation of the membrane potential. A similar tolerance toward the R groups is not a physiological requirement for the Na⁺-dependent citrate transporter CitS that functions as a Na⁺/H⁺ symporter (14–16). Consistent with this observation, the results in Table I show that CitS has a very narrow substrate specificity (mainly citrate), whereas MleP and CitP transport a wide range of 2-hydroxycarboxylates with different R substituents. Remarkably, the R groups of the transported substrates range in size from the smallest possible, *i.e.* hydrogen atoms in glycolate, to the R groups of the physiological substrates, *i.e.* malate for MleP and citrate for CitP. Larger R groups as in citromalate for MleP and isocitrate for CitP result in very poor translocation. Apparently, the translocation site is optimized for the physiological substrates, and smaller R groups are compensated for by the conformation of the protein or a varying amount of cotransported water (40, 41). In the case of CitS smaller R groups reduce transport drastically, possibly because the R groups are essential for Na⁺ binding.

The members of the 2-hydroxycarboxylate transporter family are a potent experimental system to study the relation between the primary sequence and substrate specificity. Currently, we are dissecting the binding and translocation events kinetically, analyzing the stereo selectivity of the transporters, and identifying residues in the primary sequences which are located in the binding pockets of MleP, CitP, and CitS. Such studies will eventually give a detailed model of the binding sites and explain how the details in similar structures result in important functional differences.

Acknowledgments—We thank K. Leenhouts for kindly providing *L. lactis* strain LL108, B. Poolman for initiating the collaboration between

S. D. and J. L., and W. N. Konings for carefully reading the manuscript and for many helpful suggestions.

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